PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
A01H 5/00, C12Q 1/68

A1

(11) International Publication Number: WO 92/21229

(43) International Publication Date: 10 December 1992 (10.12.92)

(21) International Application Number: PCT/US92/04593

(22) International Filing Date: 5 June 1992 (05.06.92)

(30) Priority data:
710,428 7 June 1991 (07.06.91) US

(71) Applicant: THE UNITED STATES OF AMERICA, represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; National Institutes of Health, Office of Technology Transfer, Patent Branch, Bethesda, MD 20892 (US).

(72) Inventors: BURKHART, James, G. ; 7713 Jenks Road, Apex, NC 27502 (US). MALLING, Heinrich, V. ; Route 8, Box 64C, Chapel Hill, NC 27504 (US).

(74) Agents: SCOTT, Watson, T. et al.; Cushman, Darby & Cushman, Ninth Floor, 1100 New York Avenue, N.W., Washington, DC 20005-3918 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent).

Published
With international search report.
With amended claims.

(54) Title: ΦX174 TRANSGENIC MAMMALS

(57) Abstract

The present invention relates to a transgenic non-human mammal, such as a mouse, all of whose germ cells and somatic cells contain a stably integrated DNA viral shuttle vector ΦX174, which vector is introduced into said mammal, or an ancestor of said mammal, at an embryonic stage. The invention also relates to methods of detecting mutations at the DNA level in germinal and somatic cells of non-human mammals, where mutations are detected by specific reversions from a mutant to normal phenotype.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	ML	Mali
AU	Australia	FR	France	MN	Mongolia
BB	Barbados	GA	Gabon	MR	Mauritania
BE	Belgium	GB	United Kingdom	MW	Malawi
BF	Burkina Faso	GN	Guinea	NL	Netherlands
BG	Bulgaria	GR	Greece	NO	Norway
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	RO	Romania
CA	Canada	IT	Italy	RU	Russian Federation
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland			SN	Senegal
CI	Côte d'Ivoire	KR	Republic of Korea	SU	Soviet Union
CM	Cameroon	LI	Liechtenstein	TD	Chad
CS	Czechoslovakia	LK	Sri Lanka	TG	Togo
DE	Germany	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		
ES	Spain	MG	Madagascar		

ϕX174 TRANSGENIC MAMMALS

BACKGROUND OF THE INVENTION

Technical Field

The present invention relates to a transgenic non-human mammal whose chromosomal genome is stably integrated with double-stranded DNA viral vector ϕX174. The present invention also relates to methods of detection of *in vivo* mutation.

Background Information

In general, the evaluation of spontaneous and induced mutation in mammals, for basic research endeavors or as required in regulation and license of commercial or therapeutic chemicals, has been fraught with problems and inefficiencies. Such research has typically required large numbers of animals and resulted in considerable expense. In addition, there has been significant disparity between *in vitro* tests and whole animal assays. The variation in response should be considered as a natural consequence of comparisons between different organisms, different gene products both within and between indicator species, and the potential influences of metabolism on molecular exposure and identification of the mutant phenotype. In short, for an adequate understanding of the biological complexities of cellular exposure, replication, repair, transmission and development, an analysis should be made directly at the DNA level.

The present invention provides a novel vehicle for the detection and study of *in vivo* mutation directly at the DNA level in somatic and germinal tissue using simple techniques and very

few animals. The approach has been to utilize a small DNA viral vector, ϕX174, stably integrated into the chromosomal genome of transgenic mice. There is no expression or selection of the vector's mutant or normal phenotype in the host cells or tissue. The viral vector can be recovered from the DNA of the host, and mutations within genes of the vector can be detected and analyzed. The use of the same vector can be expanded to a variety of eukaroytes and prokaryotes.

TNO Medical Biological Laboratory (Rijswijk, the Netherlands) has recently developed a transgenic mouse system for the study of mutation based on incorporation of lambda phage containing the lacZ gene. These mice are being marketed through Hazelton (Kensington, Maryland) in the United States. However, there are some fundamental differences between approaches using lambda-based vectors and the use of transgenic ϕX174.

In earlier experiments, linear ϕX174 containing the am3 mutation was cotransfected into TK mouse L cells to form a cell line containing 5 potentially recoverable ϕX copies stably integrated in a single array into the chromosomal genome. The inserted ϕX DNA was shown to be unmethylated. (Burkhart et al, Mutation Research, 213:125 (1989)) Using the cell line, methods were developed to recover to ϕX phage from the host genome. Development of methods at this stage included partial purification of the ϕX sequence by digestion of the chromosomal DNA with Sau3A I, Pvu II, and Pst I followed by S-1000 column chromatography (Sau3A I and Pvu II cleave the host DNA to an average size of about 200 bp; Pst I cleaves the linear ϕX array into single full

length φX sequences). Column chromatography was chosen over recovery from agarose gel because of the amount of DNA that can be loaded onto a column, and to avoid any use of ethidium bromide or contamination from markers. Transfection was also done of the φX into highly competent spheroplasts (1-5 x 10^{-3} per copy). Methods were developed to produce and store the spherophasts for as long as 3 years with no loss of competency. The φX transgenic cell line was treated with 20mM ethylmethane sulfonate (an alkylating agent), and it was determined that mutations could be detected as reversions of am3 among recovered phage. Overall, these previous experiments demonstrated that φX could be stably integrated into a host mammalian cell genome and efficiently recovered and that mutations could be detected among phage recovered from treated cells. (Burkhart et al, Mutation Research, 213:125 (1989).)

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a transgenic non-human mammal, such as a mouse, all of whose germ cells and somatic cells contain a stably integrated DNA viral vector φX174, which vector is introduced into the mammal, or an ancestor of the mammal, at an embryonic stage.

It is another object of the present invention to provide a method of detecting mutations at the DNA level in germinal and somatic cells of non-human mammals (such as a mouse) comprising the steps of:

(i) stably integrating the germinal and somatic cells of a non-human mammal with a vector comprising φX174 DNA and a marker gene (such as

am3, lacI, am16, am18, strp resistance or sup+ F), to produce a transgenic mammal;

(ii) isolating genomic DNA from the mammal;

(iii) partially purifying φX174 DNA from the isolated genomic DNA of step (ii);

(iv) transfecting by electroporation bacterial cells with the partially purified φX DNA of step (iii) and replicating φX phage, which bacterial cells are selected for resistance to φX infection; and

(v) growing replicated φX phage of step (iv) on selective and non-selective bacterial host strains to identify mutations.

It is a further object of the present invention to provide a method for detecting mutations at the DNA level of a non-human mammal, using the above-described method, where the bacterial cells of step (iv) are developed from E. coli C. and have the characteristics of being resistant to infection by φX phage and exhibiting high transfection efficiency for φX by electroporation.

It is yet another object of the present invention to provide a method for detecting mutations at the DNA level of a non-human mammal, using the above-described method, where the bacterial host strain of step (v) is E. coli.

Another object of the present invention is to provide a method for detecting mutations at the DNA level of a non-human mammal, using the above-described method, where the mutations are detected by specific reversions from a mutant to normal phenotype.

Another object of the present invention is to provide a method for detecting mutations at the DNA level of a non-human mammal, using the

above-described method, where step (v) further comprises deriving the total number of rescued progeny ϕX174 phage and the number of mutants.

It is another object of the present invention to provide a transgenic non-human mammal as described above, where the vector is a shuttle vector.

Further objects and advantages of the present invention will be clear from the description that follows.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: ϕX Copy Number in Founder Animal #37. Pst 1 digestion of genomic DNA from a founder animal (#37) followed by electrophoresis with prepared standards of mouse DNA containing ϕX RFDNA in known concentrations indicates that approximately 50 copies had been integrated into the mouse genomic DNA. Of the first 4 founders animals, all had approximately the same number of copies.

Figure 2: ϕX Copies on Homozygous 54 Line Mice. Pst 1 digestion of DNA from subsequent breeding of founder 54 indicates that the line is homozygous for the vector and that there are about 100 copies of the ϕX sequence incorporated into the mouse genome.

Figure 3: ϕX Line 54. Transmission and litter data from line 54 mice indicates that the vector does not appear to have any detrimental or recessive lethal effects. (The normal size for a C57BI6/J litter is 6.5.)

Figure 4: Methylation of ϕX174 in Transgenic Mouse Line 54. Digestion of ϕX RFDNA

and genomic DNA from transgenic mice with Hpa II (methylation sensitive) and Msp (methylation insensitive) indicates that the vector DNA is methylated in the host genome.

Figure 5: Recovery of ϕX174 from a Transgenic Mouse with Approximately 50 Potentially Active Copies. Example of phage recovery from the mouse genome demonstrates sufficient numbers to allow mutation analysis.

Figure 6: am3 Reversions in Chromosomally-Integrated ϕX Induced by N-Ethyl N-Nitrosourea in Mouse Liver. Analysis of mutations at am3 in phage recovered from untreated mice, and mice treated with 200 mg/kg n-ethyl-n-nitrosourea indicates a 5-fold increase in mutation induction in somatic tissues of treated animals.

Figure 7: The Overall Scheme for the Study of in vivo Mutagenesis in ϕX Transgenic Mice.

Figure 8: Construct of ϕX174.

DETAILED DESCRIPTION OF THE INVENTION

In one embodiment, the present invention relates to a transgenic non-human mammal (for instance, a mouse) all of whose cells, including germinal and somatic cells, contain a stably integrated DNA viral vector ϕX174. The present invention also relates to the stable integration of DNA vector ϕX174 into the genome of an ancestor of the non-human mammal. The vector is introduced

into the mammal at an embryonic stage, by methods well-known in the art.

Using a shuttle-vector approach, the Applicants have developed a method for the study of mammalian mutagenesis by devising a small vector (5.3 kb in length) that has been stably integrated into the chromosomal genome of mammals (i.e. transgenic mice) with no expression of ϕX in the host and no detectable homology between the ϕX sequence and the mammalian genome. Further, there was no selection of vector normal or mutant phenotype in the host cells or tissue.

In this invention, viable phage can be efficiently recovered from the chromosomal DNA of the host mammal, and mutations can be detected among phage recovered from treated cells. Mutations are not significantly induced by the recovery process. Additionally, there is an increase in the mutation frequency of the vector after treatment of the host with a known mutagen.

In another embodiment, the present invention relates to a method of detecting mutations at the DNA level in germinal and somatic cells of non-human mammals (for instance, mice) comprising the steps of:

(i) stably integrating the germinal and somatic cells of a non-human mammal with a vector comprising ϕX174 DNA and a marker gene, to produce a transgenic mammal;

(ii) isolating genomic DNA from the mammal;

(iii) partially purifying ϕX174 DNA from the isolated genomic DNA of step (ii);

(iv) transfecting, advantageously by electroporation, bacterial cells with the partially purified ϕX DNA of step (iii) and

replicating ϕX phage, which bacterial cells are selected for resistance to ϕX infection; and

(v) growing replicated phage of step (iv) on selective and non-selective bacterial host strains to identify mutations. As contemplated by the invention, the vector is a shuttle vector, and mutations are detected by specific reversions from a mutant to normal phenotype. The marker gene of step (i) may be of the group comprising am3, lacI, am16, am18, strp resistance and sup+ F.

The bacterial cells of step (iv) may be taken from a strain developed from E. coli C. (designated CϕXR1), and act effectively as a recovery mechanism of ϕX. These cells have been developed to be resistant to infection by ϕX phage particle and exhibit high transfection efficiency for ϕX by electroporation. Hence, there is little or no chance for the recovery of ϕX DNA sequences from host genomic DNA to be contaminated. For instance, contamination could occur by wild type ϕX, which would negate the accuracy of any mutation detection data. Likewise, there is little or no chance for reinfection by ϕX from accidental lysis of bacterial cells.

The recovery E. coli strain CϕXR1 does not contain restriction enzymes that cleave DNA that has been CpG methylated, unlike immortal mammalian cell lines. Notably, ϕX in transgenic cell lines is not methylated, whereas ϕX in the transgenic mammals of the present invention is methylated.

A sample of the strain CϕXR1 has been deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852. The deposit number is _____. Deposit is not intended to limit the concept of the present invention to the particular biological

material deposited. The deposit will be maintained according to applicable laws, and will be available to the public as required.

The bacterial host strain of step (v) may be E. coli. In addition, step (v) may further comprise deriving the total number of rescued progeny φX174 phage and the number of mutants.

The present invention demonstrates a number of significant advantages over the prior art use of lambda-based vectors. For instance, the integrated lambda cloning vector is approximately 50 kb in length, whereas φX is 5 kb. The small size of φX means that many more copies can be inserted into the host genome with less overall potential for genomic disruption or reduction in viability. The smaller size of the φX vector also means that there is less likelihood for cleavage (or rearrangement) of the vector sequence during recovery from the host or in any subsequent analysis. Finally, the common use of lambda-based vectors as a standard laboratory cloning vehicle demonstrates that in use as mutation shuttle vectors, they may be subject to internal rearrangement independent of actual mutation events in the host. Size-related fragmentation and rearrangement would cause misrepresentation of mutations occurring in the host organism. In addition, there is evidence for the existence of some regions of homology between lambda and mammalian genomes; there is no such homology between φX and mammalian genome. (Cohen et al, Gene 69:131 (1988))

Secondly, mutation detection is superior in the present invention. Mutations are currently being detected in λ shuttle vectors as forward events. In φX174 vectors, mutations are detected as specific reversions. Forward mutation

detection in lambda means that the target sequence is large (in the range of 3000bp). But in order to be detected, the mutation must inactivate the translation product. All viable lambda must be screened histochemically and the nature of any putative mutation can only be determined by sequence analysis. Although the large target size associated with forward mutation detection is somewhat desireable because of the potential number of mutations, the indicator inactivation is not specific within the target gene. Furthermore, forward mutation detection tends to have a high background and can be biased towards mutations producing internal stop codons, frame shifts, or sequence rearrangement. This high background makes analysis of germinal mutations and low-frequency somatic mutations very difficult.

In ϕX174 vectors, however, mutations are detected at specific base pairs by reversion from a mutant to normal phenotype. The spectrum of mutation for reversion is limited, but the exact event is well defined by selection. Sequence analysis is not necessary, and the inventors have never observed a false positive in the reversion assay. A low background is important if the investigator seeks to gain insight into germinal transmission of mutation or repair. The mutant detection method used in the ϕX174 transgenic system also allows the investigator to distinguish mutations that occurred in the host from those that arose as <u>de novo</u> events after excision of the vector from the host. Background and spontaneous mutations are very low, and certain events not usually observable in a forward mutation system (such as transitions at an A:T pair) can be seen.

Comparison of mutation data from forward mutation systems in shuttle vectors and <u>in vivo</u>

transmissable mutation data after treatment with N-ethyl-N-nitrosourea (ENU) provide some insight into the problems associated with the singular reliance on a forward detection system. ENU is a general alkylating agent and potent mammalian mutagen. Analysis of mutations detected in a shuttle vector after treatment of transgenic mice indicates that all detected substitutions occur at a G:C base pair. In contrast, all of the mutations recovered from the biochemical specific locus test in mice, which identifies mutants on the basis of altered protein structure, have been induced at the A:T base pair.

Identification of the specific event in forward mutation detection requires sequence analysis, and sequencing often requires production of a single stranded template. Such analysis is more easily accomplished with ϕX than a lambda phage because the packaged ϕX phage (virion) rescued from the host DNA is naturally single stranded. Phage lambda does not have a single stranded stage.

Thirdly, the ϕX174 vector system facilitates better recovery of the phage DNA. Lambda phage are recovered from host DNA by electrophoretic purification of the phage sequence followed by packaging of the phage DNA into virions using highly specialized cell extracts. These cell extracts are difficult to produce and, when purchased from a vendor, may constitute a considerable expense for any laboratory using the lambda vector. In contrast, ϕX174 can be easily partially purified from the host organism by restriction enzyme digestion of the host genomic DNA and a simple modification of currently available mini-column technology, without any requirement for electrophoresis or probe

hybridization to identify the specific sequence. The phage can then be recovered by electroporation into an E. coli strain developed to be resistance to infection by the ϕX phage particle and to exhibit high transfection efficiency for ϕX by electroporation. The competency of the eletroporation protocol is in the range of 10-20%. Recovery of lambda and ϕX174 are comparable per genomic copy.

The ϕX174 system is also advantageous in the production of the transgenic strains. The commercially available transgenic strains carrying lambda vectors have been produced by injection of F1 hybrid ova in order to take advantage of enhanced vigor of the eggs produced in such crosses. From a genetic standpoint this is somewhat undesirable because: 1) although the transgenic insert can be made homozygous in a particular line, all other genes that differ in the two parental types will assort independently; thus, a parental type homozygous for the insert cannot be reproduced; 2) each line produced by brother-sister matings of the F1 generation from the transgenic founder will constitute a recombinant inbred line; 3) data from whole animal studies using biological markers in response to toxicological exposure indicates that such lines can be very different in response both from one another and from the parent strains due to segregation of genes important in the processes of xenobiotic metabolism. The combination of inbred and independent mutation markers in the method of the present invention means that single lines can be constructed with multiple specific markers, and a forward ϕX mutation marker can also be developed. The skilled artisan would then be able to distinguish between various types of

induced mutation events in a single mouse without the a priori requirement for sequence analysis of each mutant.

The present invention can be illustrated by the use of the following non-limiting examples.

Example 1

Production of Transgenic Mice

ϕX174 shuttle vector is constructed using methods well known in the art, according to figure 8. Purified ϕX RFDNA is linearized by digestion with Pst I, then chloroform-phenol extracted and ethanol precipitated. In order to increase the copy number per insertion site, the linear ϕX DNA is ligated at various short (i.e. 30 sec-5 min) intervals with T4 DNA ligase or E. coli DNA ligase. Aliquots of the ligated DNAs are examined by agarose gel electrophoresis and ethidium bromide staining; a sample is selected based on the existence of a ladder of catenated ϕX copies between 2 and 10. The fertilized mouse ova are then injected with 100-250 copies of the ϕX RFDNA according to standard procedures available in Hogan et al, "Manipulating the Mouse Embryo" Cold Spring Harbor, (1986). Genomic DNA from tail clips of mice resulting from the injected ova are screened for the presence of ϕX sequences after Pst I digestion. For any mouse containing the sequence, the number of copies and insertion sites are estimated (see enclosed figures). The transgenic founders are mated and the progeny similarly screened. Transgenic males and females from the same founder are subsequently mated to produce a line of mice homozygous for the particular ϕX insertion.

Example 2

Isolation of genomic DNA

Various tissues may be excised and used immediately or frozen at -70°C as needed. Approximately 1 gm tissue (or less as applicable) is homogenized on ice in 20mL cold 0.25M sucrose-TE (10M Tris, 1mM ETA, pH 7.6) using a polytron (Brinkman or equivalent). The homogenate is layered over 20ml cold 0.35M sucrose-TE and centrifuged for 10 min at 4°C and 3000rpm in a standard preparative centrifuge (IEC or equivalent) equipped with a swinging bucket rotor. The supernatant is discarded and 5mL proteinase K (2mg/mL) in 50mM Tris, 100mM EDTA, 100mM NaCl, 1% SDS, pH 8.0 is added to the pellet and vortexed. The suspension is incubated ta 55°C for 2hr. with gentle mixing, then extracted twice with an equal volume chloroform-phenol (1:), twice with chloroform, then ethanol precipitated. The DNA is suspended in TE at a final concentration in the range of 1μg/μL.

Example 3

Partial purification of ϕX ds DNA

Minicolumns containing Sephacryl S-1000 (Pharmacia) that has been filtered, equilibrated with TE, autoclaved and thoroughly degassed are prepared in advance. For convenience, these columns should be compatible with a pipetter such as the Rainin 1000. 100μg host DNA is digested simultaneously with 100U each PST I, PVU II, and SAU3A 1 at 37°C for 3hr with gentle mixing. The restriction enzymes are removed by proteinase K digestion (19μg) at 55°C for 1 hr followed by extraction nd precipitation as described. The pellet is resuspended in approximately 25-50μL TE and warmed to insure that the DNA is in a

homogenous solution. The DNA is loaded onto the minicolumn and eluted with 1mL TE. Using a 10X stock solution, the eluent is made compatible for T4 DNA ligase assay conditions (not containing PEG); 4-10U ligase is added and the solution is incubated at approximately 12°C for 2-3 hr. The ligation is stopped by heating to 65°C for 10 min and exchanging the solution 3 times against sterile filtered after using 300,000 nmwl miniature filters and centrifugation. The final volume reduction is suspended in 500µl water. We are constantly making improvements in this area, and there is a possibility for removing the S-1000 column step and still maintaining recovery.

Example 4

Transfection by electroporesis

Cells for electroporation are prepared in advance and stored according to this general protocol. The particular culture for electroporation of recovered ϕX174 ds DNA has been developed from E.coli C by selection for resistance to ϕX infection and as a fast growing clone in NZY medium. A log culture can be prepared, and DMSO added to a final concentration of 7%. The culture is aliquoted and frozen at -70°C until use. The cultures for preparation of competent cells are started by inoculating 50mL of NZY medium with a very small amount of cells from the frozen stock culture. This culture is grown overnight with vigorous shaking. A second overnight culture is then prepared from this culture by inoculating the same amount of medium with a very small amount (100µL) of the first overnight culture. For the final culture, 1 liter NZY medium in a 4 liter erlenmeyer flask is inoculated with the second overnight culture to a

final density of OD_{600}=0.05. The cultures are incubated at 37°C with shaking (300rmp) until OD_{600}=0.5. At this point, the media are cooled down as quickly as possible in ice water. After the growth phase is completed, the cells are concentrated by centrifugation and washed thoroughly in sterile distilled water. Finally, the cells are suspended in 10% glycerin. The concentration of the cells is adjusted to 2.5 x 10^{10} cells per ml; the cell suspension is aliquoted into eppendorf tubes, quickly frozen in liquid nitrogen and stored at -70°C. Although cultures for preparation of competent cells for transfection by electroporesis are usually grown in various complete media, the inventors have found that the concentration of bi-valent cations (metals) has a significant influence on the competence of the cells. Among many different complete media, an NZY medium which contains only magnesium ions has been found to be far superior.

Approximately 1 hr before transfection with the purified DNA, the cell suspensions are removed from the -70°C freezer and placed on ice. 300μL of cell suspension is aliquoted into an eppendorf tube, 100μL of the DNA solution in water is added, and 400μL are pipetted into a disposable electroporesis cuvette. The electroporesis is done at 2.5kv with a resistance setting of 246 ohms. Immediately following the electroporesis, the transfected cell suspension is suspended in SOC media, incubated for 2 hours with vigorous shaking. A lysis buffer is added together with lysozyme (final conc = 5mg/mL). The culture is left overnight in the refrigerator at 4°C, then diluted and plated as standard for φX174 on selective and non selective E.coli host strains to

derive the total number of rescued progeny phage and the number of mutants.

Example 5

Preparation of indicator cultures.

1. Cultures for Determination of Total Phage Titer: An o/n culture of CQ-2 is prepared from the frozen stock cultures by scraping a little of the surface of the frozen culture with a plastic 1 ml pipet. It is not necessary to defrost the stock culture. The scraping is added to 100 ml LKC medium in an 500 ml erlenmeyer flask an incubated at 37°C o/n on an airshaker at 300 rpm. From the o/n culture is prepared a log-culture by pipetting 1 ml of the o/n culture into 100 ml LKC medium and place on the same airshaker as the o/n culture. When OD^{600} reaches 0.3-0.5 the culture is placed on ice. This culture can be used for several days.

2. Cultures for Determination of am3 Reversions. The am3 reversions is determined by plating the phage suspension on E. coli C. That baterial strain does not contain any suppressor and is therefore selective for am3 reversions. The plating cultures are prepares as the CO-2 culture.

Example 6

Dilution of Samples.

1. Dilution of the samples in order to determine the total liter. Pipet 1 ml of the electroporesis sample after lysis into a 1.5 ml eppendorf tube with screw cap. Dilute by sequentially add 100 µl to 1.5 ml eppendorf tubes with 0.9 ml 0.05 M Sodium tetraborate at pH 8.0. It is practical to dilute a total 4 sequential times; the last dilution is now 10^{-4} of the original.

2. Dilution to determine the am3 reversion frequency. The am3 reversion are determined from plating of the undiluted samples or from the 10^{-1} dilution tube.

Example 7

Plating of the Samples.

1. Plating of the samples in order to determine the total titer. One ml of the log CQ-2 indicator culture is pipetted into a test-tube and placed in a 37°C circulating waterbath. 20 µl of the sequential dilutions are added to each of the test-tube containing the indicator culture; incubated approximately 3 min. then 2-5 ml soft agar is added; and the content are mixed slightly on a vortexes and plated on bottom agar. The plates are plated in a single layer on the shelfs in a 37° incubator.

Every day a plating is done the CQ-2 culture is checked for its infectivity and possible contamination with spurious phages. The infectivity check is done by plating 20 µl of a phage suspension which contain 40-80 infective centres. It is practical to make many individual stock cultures of ΦX174 am3 cs70 in borate is stable in the coldroom at 4°C for several years. The presence of spurious phages in the bacteria culture is done by plating 4 x 1 ml of the bacteria culture as described above but without any addition of phages.

2. Plating of the samples to determine the am reversion frequency. Ten ml of the E. coli C log culture is added to a test-tube and placed in the 37°C circulating waterbath. After a couple of min. when the culture is temperature equilibrated o.5 ml of the undiluted lysis from

the electroporesis culture is added. After further incubation of 2-3 min. the content is mixed with 4 ml of 1.6% top agar and plated on a 150 mm petriplate with bottom agar. These plates have been preheated to 37°C. Normally the total sample is plated.

Every day a plating is done to determine the am3 reversion frequencies the E. coli C culture is checked for infectivity and contamination with spurious phages. The check for infectivity is done by using the ΦX174 wild type phage. The cs70 mutant serves as a check on the genotype of the revertants detected on the plates from the DNA from the animals. The wildtype does not contain the cs70 mutant. The infectivity is checked by adding .5 ml of a phage suspension which contain approximately 50-100 infective centres following the procedure for plating a determine the reveres mutation frequency. Plating for the check of presence of spurious phages is done by plating 4 x 10 ml bacteria solution without addition pf phages.

Example 8

Calculations.

1. Total Number of ΦX174 in the Sample.

The total number of phages in the sample equal:

$$\frac{\text{(Number of plagues on CQ-2) x 15 x 1000 x (dilution factor}}{(\mu l \text{ plated})}$$

15 is the total volume of the electroporesis culture. μl plated is usually 20μl.

2. Competence of the Cells.

The competence of the cells can in general be described as the efficiency by which the ΦX174 DNA has entered the bacterial cells. The competence is proportional to the concentration of the DNA within many orders of magnitude. Nevertheless we

have selected a concentration of ΦX174 RF DNA (double standard) which yield the same number of phages as can be expected from a good DNA sample from the animals. Competence equal:

$$\frac{\text{Total number of phages in the same X 100\%}}{2 \times 10^{8}(=\text{molecules}) \times 100(=\text{burst size})}$$

3. Total Number of Recovered Progeny in the Sample. The burst size of the ΦX174 is approximately 100. The total number of recovered progeny from the mammalian is therefore equal to the total number of phages in the sample divided with the burst size.

4. Total Number of Viable ΦX174 Molecules in the Sample from the Animals. The total number of recovered progeny in the sample from the animal depends on the competence. In order to calculate the total number of viable molecules obtained from the animals divide the number in "3" with the competence.

5. Calculation of Reverse Mutation Frequencies. The revers mutation frequency equal:

$$\frac{\text{Total number of revertants in the sample}}{\text{Total number of phages in the sample}} \times 10^{8}$$

It is often practical to express the reversion frequencies with the multiplication factor of 10^{8}. Therefore the factor 10^{8} in the equation

The overall scheme for the study of <u>in vivo</u> mutagenesis in ϕX transgenic mice is provided in the enclosed figure 7. The actual detection of mutations among ϕ phage rescued from a host animal is accomplished by simple techniques or modifications following this general example. Techniques in molecular biology are continuously improving; these should only be regarded as

current examples used to accomplish the required steps.

All publications mentioned hereinabove are hereby incorporated by reference. While the foregoing invention has been described in some detail for purposed of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention.

WHAT IS CLAIMED IS:

1. A transgenic non-human mammal all of whose germ cells and somatic cells contain a stably integrated DNA viral vector φX174, which vector is introduced into said mammal, or an ancestor of said mammal, at an embryonic stage.

2. The mammal of claim 1, wherein said mammal is a mouse.

3. A method of detecting mutations at the DNA level in germinal and somatic cells of non-human mammals comprising the steps of:

(i) stably integrating the germinal and somatic cells of a non-human mammal with a vector comprising φX174 DNA and a marker gene, to produce a transgenic mammal;

(ii) isolating genomic DNA from said mammal;

(iii) partially purifying φX174 DNA from the isolated genomic DNA of step (ii);

(iv) introducing into bacterial cells the partially purified φX DNA of step (iii) and replicating φX phage, which bacterial cells are selected for resistance to φX infection; and

(v) growing the replicated φX phage of step (iv) on selective and non-selective bacterial host strains to identify mutations.

4. The method according to claim 3 wherein the introduction of step (iv) is effected by electroporation.

5. The method according to claim 4, wherein the bacterial cells of step (iv) are developed from E. coli C. and have the characteristics of being resistant to infection by

ϕX phage and exhibiting high transfection efficiency for ϕX by electroporation.

6. The method according to claim 3, wherein the bacterial host strain of step (v) is E. coli.

7. The method according to claim 3, wherein said non-human mammal is a mouse.

8. The method according to claim 3, wherein said mutations are detected by specific reversions from a mutant to normal phenotype.

9. The method according to claim 3, wherein said marker gene of step (i) may be of the group comprising am3, lacI, am16, am18, strp resistance and sup+ F.

10. The method according to claim 3, wherein step (v) further comprises deriving the total number of rescued progeny ϕX174 phage and the number of mutants.

11. The mammal according to claim 1, wherein said vector is a shuttle vector.

12. The method according to claim 3, wherein said vector is a shuttle vector.

13. The method according to claim 3, wherein said the bacterial cells of step (iv) have the ATCC accession number _____.

AMENDED CLAIMS

[received by the International Bureau on 7 October 1992 (07.10.92);
original claims 3,5 and 10 amended; new claim 14 added;
remaining claims unchanged (3 pages)]

1. A transgenic non-human mammal all of whose germ cells and somatic cells contain a stably integrated DNA viral vector ϕX174, which vector is introduced into said mammal, or an ancestor of said mammal, at an embryonic stage.

2. The mammal of claim 1, wherein said mammal is a mouse.

3. A method of detecting mutations at the DNA level in germinal and somatic cells of non-human mammals comprising the steps of:

(i) stably integrating the germinal and somatic cells of a non-human mammal with a vector comprising ϕX174 DNA and a marker gene, to produce a transgenic mammal;

(ii) isolating genomic DNA from said mammal;

(iii) partially purifying ϕX174 DNA from the isolated genomic DNA step (ii);

(iv) introducing into bacterial cells the partially purified ϕX DNA of step (iii) and replicating ϕX phage from the ϕX DNA, which bacterial cells are pre-selected for resistance to ϕX infection; and

(v) growing the replicated ϕX phage of step (iv) on selective and non-selective bacterial host strains to identify mutations.

4. The method according to claim 3 wherein the introduction of step (iv) is effected by electroporation.

5. The method according to claim 4, wherein the bacterial cells of step (iv) are developed from E. coli C. and have the characteristics of being resistant to infection by ϕX phage and exhibiting transfection efficiency for ϕX by electroporation in the range of 10-20%.

6. The method according to claim 3, wherein the bacterial host strain of step (v) is E. coli.

7. The method according to claim 3, wherein said non-human mammal is a mouse.

8. The method according to claim 3, wherein said mutations are detected by specific reversions from a mutant to normal phenotype.

9. The method according to claim 3, wherein said marker gene of step (i) may be of the group comprising am3,lacI, am16, am18, strp resistance and sup+ F.

10. The method according to claim 3, wherein step (v) further comprises determining the total number of rescued progeny ϕX174 phage and the number of mutants.

11. The mammal according to claim 1, wherein said vector is a shuttle vector.

12. The method according to claim 3, wherein said vector is a shuttle vector.

13. The method according to claim 3, wherein said the bacterial cells of step (iv) have the ATCC accession number_____.

14. The method according to claim 3, wherein the bacteria cells of step (iv) do not cleave methylated DNA.

FIG. 1

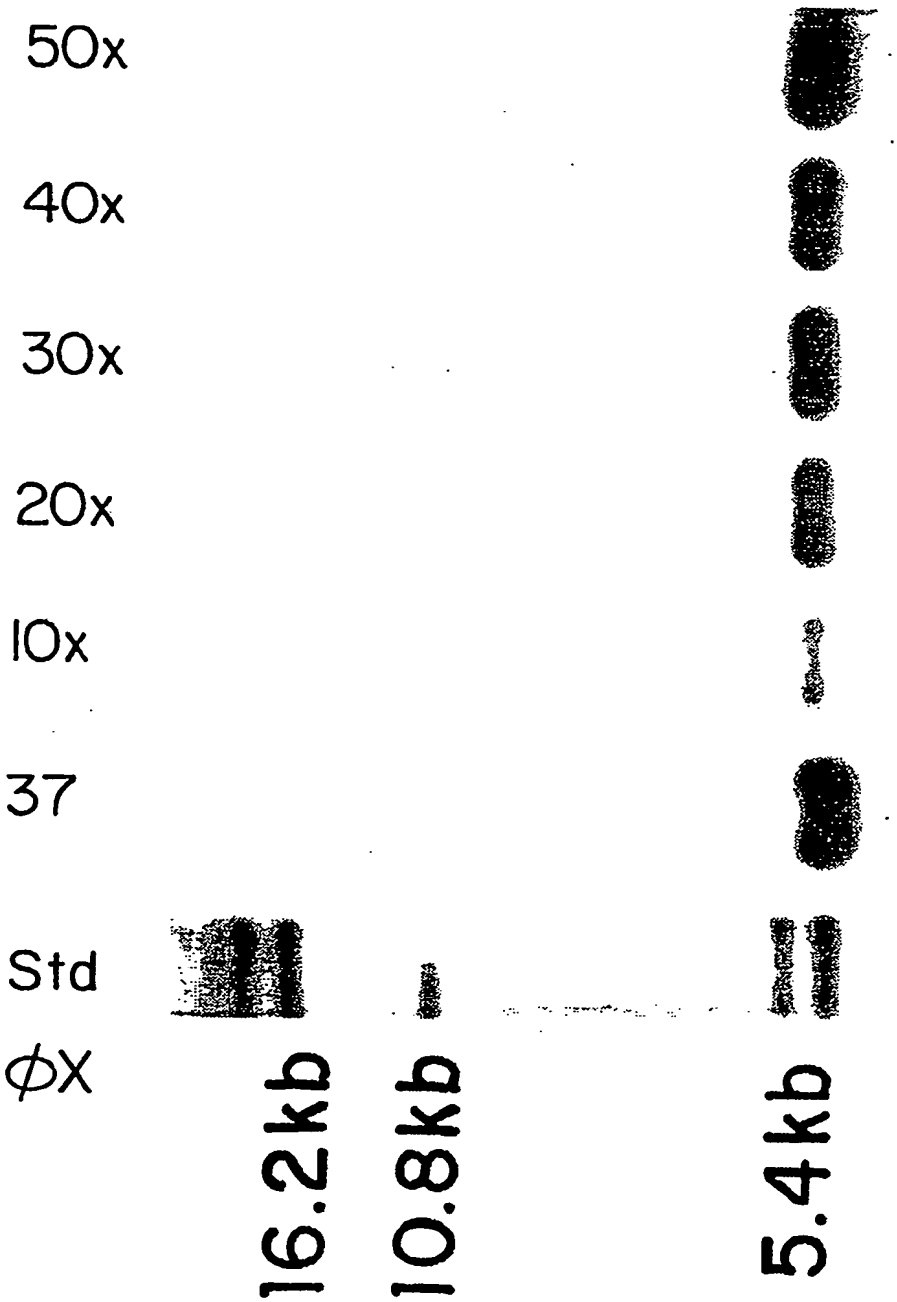
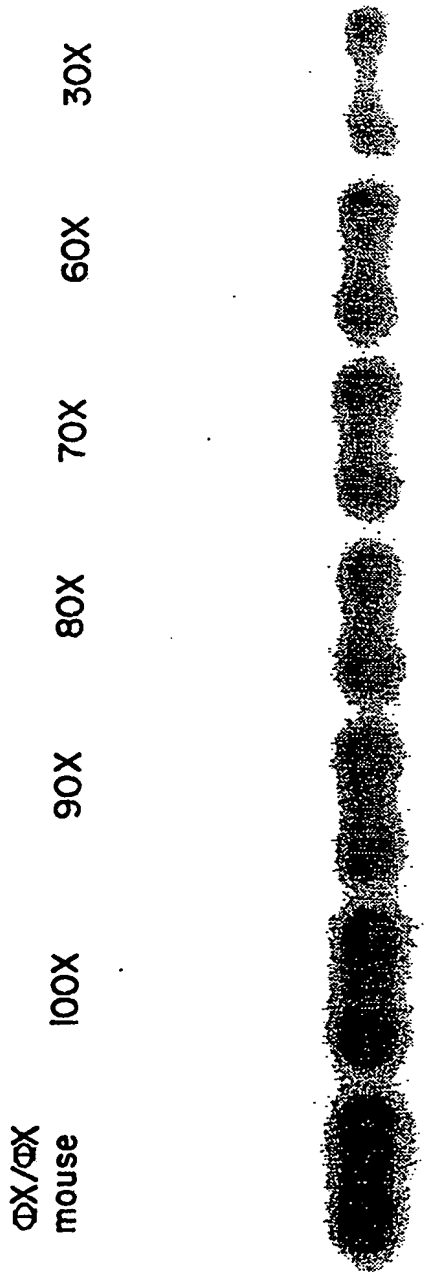


FIG. 2



SUBSTITUTE SHEET

FIG. 3

		F1			F2			F3	
Founders	Transmission Ratio	Litter Size	Transmission	F/M Ratio	Litter Size	Transmission	F/M Ratio	Litter Size	F/M
4/108	3/4	6.3(±0.5)	5/16	12/9	6.2(0.4)	11:20:21	21/23	6.7(±0.7)	25/22

FIG. 4

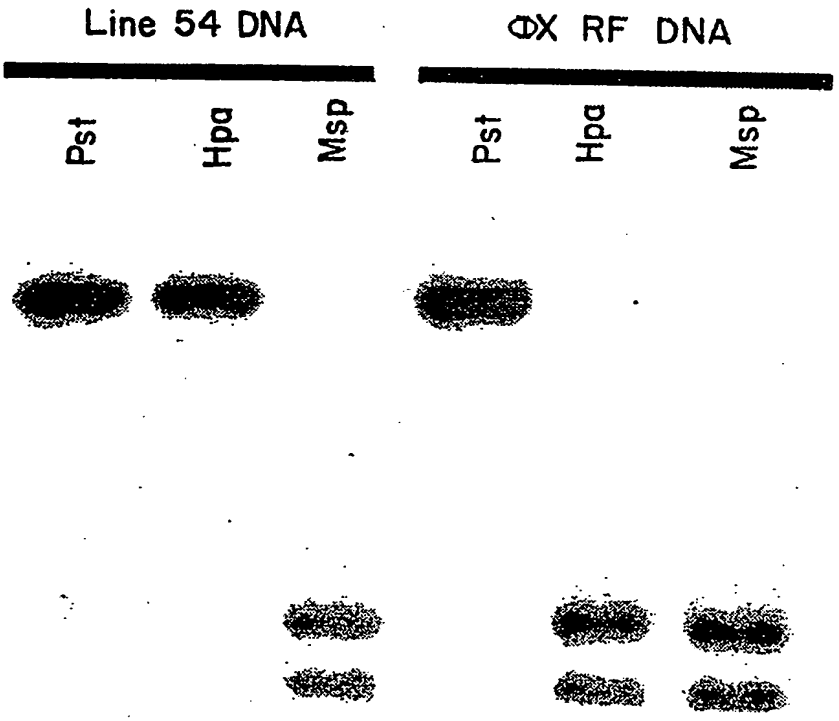


FIG. 5

Copies /µg host DNA	PFU/µg host DNA	Rescue Efficiency per phage copy	Tested Competency of spheroplasts
7.5×10^6	9.2×10^5	1.2×10^{-3}	2.0×10^{-3}

FIG. 6

GROUP	PROGENY PHAGE	am3 REVERTANTS	MUTATION FREQUENCY
Untreated	4.3×10^7	36	8.3×10^{-7}
200mg/kg ENU	1.1×10^7	52	4.6×10^{-6}

FIG. 7

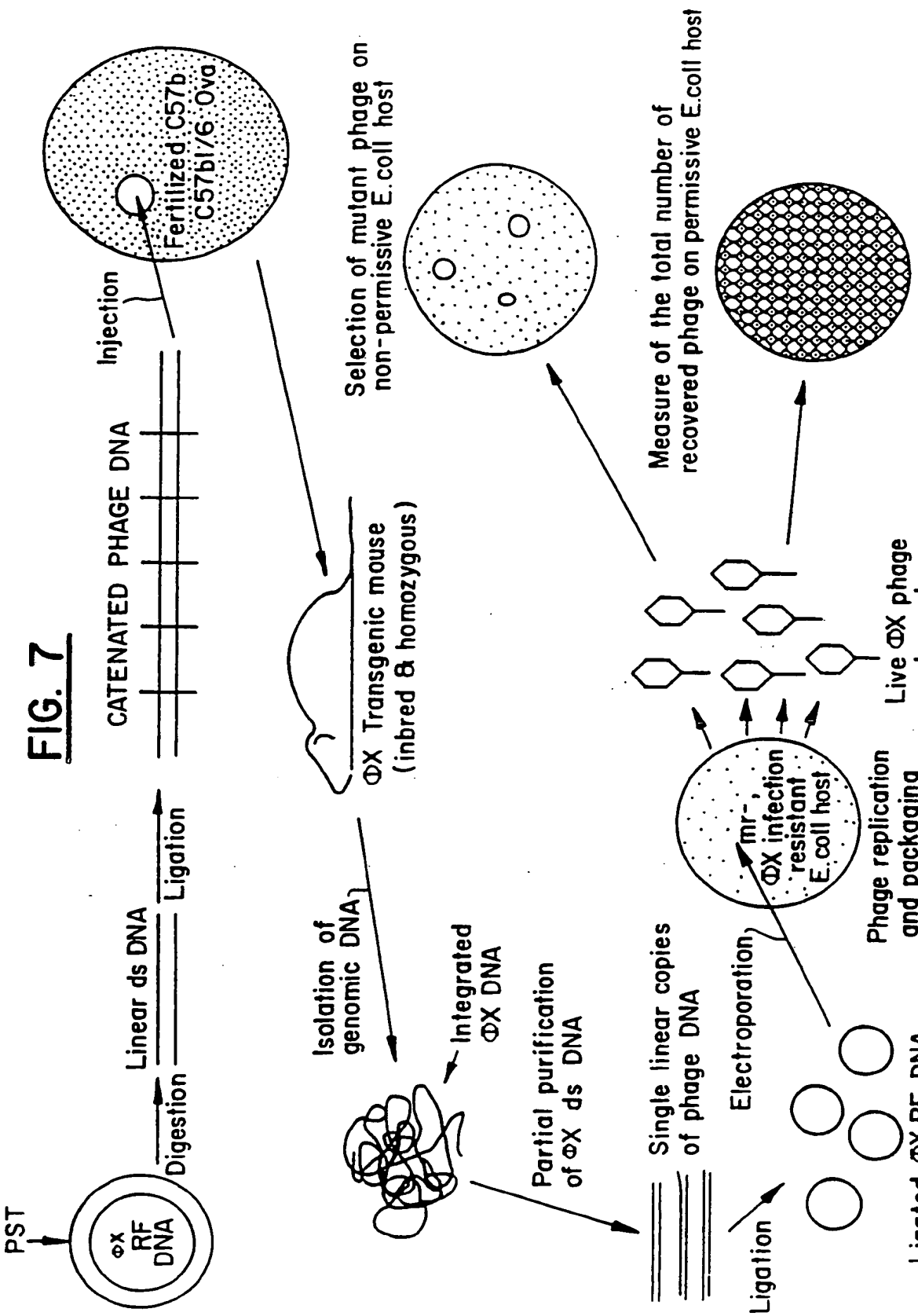
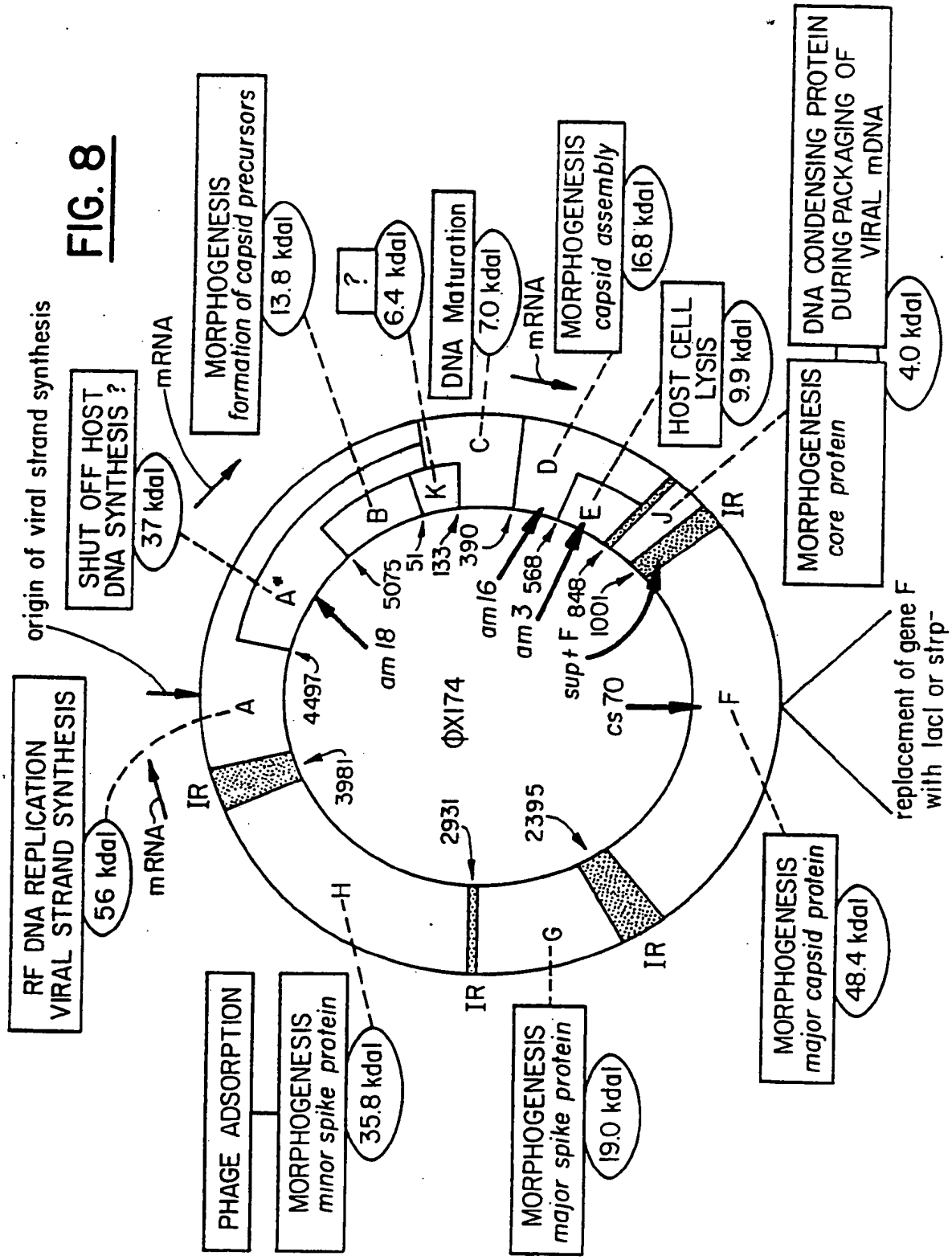

PST
ΦX RF DNA
Digestion
Linear ds DNA
Ligation
CATENATED PHAGE DNA
Injection
Fertilized C57b C57bl/6 Ova
ΦX Transgenic mouse (inbred & homozygous)
Isolation of genomic DNA
Integrated ΦX DNA
Partial purification of ΦX ds DNA
Single linear copies of phage DNA
Ligation
Ligated ΦX RF DNA
Electroporation
mr- ΦX infection resistant E.coll host
Phage replication and packaging
Live ΦX phage released
Selection of mutant phage on non-permissive E.coll host
Measure of the total number of recovered phage on permissive E.coll host

FIG. 8



SUBSTITUTE SHEET